Remarks

Reconsideration and withdrawal of the objections to the Figures and specification, and the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested. Claims 1, 4 and 12 are amended, claim 15 is canceled, and claims 32-35 are added; as a result, claims 1-14 and 32-35 are now pending in this application. The amendments are intended to advance the application and are not intended to concede to the correctness of the Examiner's position or to prejudice the prosecution of the claims prior to amendment, which claims are present in a continuation of the present application.

Amended claim 1 is supported by originally-filed claims 1 and 12.

Amended claim 4 is supported at page 16, line 29-page 17, line 1 of the specification.

New claims 32-33 are supported by the example in the specification.

New claims 34-35 are supported by originally-filed claims 17-18, respectively.

Figures 1 and 3 of the drawings were objected to as the panels in those Figures were not separately labeled. However, only Figures 1 and 2 have panels, thus substitute Figures 1 and 2 with separately labeled panels are enclosed herewith. In addition, the brief description of Figure 1 is amended to refer to each panel in that Figure.

The 35 U.S.C. § 112 Rejections

The Examiner rejected claims 1-11 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The amendments to claims 1 and 4 render this rejection moot.

The Examiner also rejected claims 1-3 and 5-11 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is respectfully traversed.

In particular, the Examiner asserts that other than a mutant MDCK cell, i.e., a MaKS cell, having decreased levels of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid compared to the parent cell line, MDCK, the instant specification fails to teach any other mammalian cell,

including a bovine cell, swine cell, simian cell, mink cell, or mink lung cell having decreased levels of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid compared to their respective parent cell line which has the ability to support efficient virus replication.

To provide an adequate written description for a claimed genus, the specification can provide a sufficient description of a representative number of species by an actual reduction to practice, reduction to drawings or by a disclosure of relevant, identifying characteristics, i.e., by a structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics. Guidelines for Examination of Patent Application under the 35 U.S.C. § 112(1) Written Description Requirement, Fed. Reg., 66, 1099 (2001). Satisfactory disclosure of a representative number of species depends on whether one skilled in the art would recognize that Applicant was in possession of the necessary common attributes or features of the elements possessed by members of the genus. Guidelines for Examination of Patent Applications under the 35 U.S.C. § 112(1) Written Description Requirement, Fed. Reg., 66, 1099 (2001). Applicant need not teach what is well-known to the art.

The specification describes the use of two lectins known to bind to sialic acid containing molecules to select for cells with reduced numbers of those molecules from parent cells known to be competent for influenza virus replication, e.g., bovine cells, swine cells, ferret cells, human cells, canine cells and avian cells, i.e., cells from organisms recognized as being susceptible to influenza virus infection (see Table 4 in Chapter 51 of Fields Virology, Knipe et al., eds., (1985), a copy of which is included herewith). Further, influenza virus is known to bind to sialyl oligosaccharides on cells (page 1, lines 17-21 of the present specification).

Accordingly, Applicant has disclosed relevant, identifying characteristics of the genus of claimed mutant cells by disclosing a functional characteristic coupled with a known or disclosed correlation between function and structure, e.g., the use of lectins which bind sialic acid to select for cells with reduced sialic acid containing molecules, molecules which are receptors for influenza virus, such that one skilled in the art would recognize that Applicant was in possession of the necessary common attributes or features of the elements possessed by members of the genus. Therefore, Applicant's specification satisfies the written description requirement of 35 U.S.C. § 112(1).

Hence, withdrawal of the § 112 rejections is respectfully requested.

The 35 U.S.C. § 102 Rejections

The Examiner rejected claims 1-4 and 8 under 35 U.S.C. § 102(b) as being anticipated by Martin et al. (Virology, 241:101 (1998)) or Brandli et al. (J. Biol. Chem., 263:16283 (1988)). These rejections, as they may be maintained with respect to the pending claims, are respectfully traversed.

Martin et al. disclose that influenza virus hemagglutinin (HA) proteins with substitutions in the receptor binding site can affect the ability of HA to bind to human erythrocytes, presumably due to the reduced affinity of mutant HA for sialic acid (pages 105-106). It is disclosed that four transfectant viruses with mutant HAs were able to infect MDCK cells and embryonated chicken eggs with efficiencies comparable to wild-type (page 106), although the infectivity of one of the transfectant viruses on a mutant ricin-resistant MDCK cell ("MDCK RCA^r") was greatly reduced compared to wild-type MDCK cells. It is disclosed that MDCK RCA^r cells have a 70 to 75% reduction in cell surface sialic acid (citing to Brandli et al., 1988), and that these cells may produce reduced virus yields (citing Green et al., J. Cell. Biol., 89:230 (1981)).

Brandli et al. disclose that a ricin-resistant MDCK cell line (MDCKII-RCA^r) and wild-type cells bind wheat germ agglutinin (specific for *N*-acetylglucosamine and *N*-acetylneuraminic acid), conconavalin A (specific for mannose) and *H. pomatia* agglutinin (*N*-acetylgalactosamine), which binding was unaffected by exogalactosylation (page 16286). It is further disclosed that wild-type cells did not contain significant amounts of *N*-acetylglucosamine (assessed by *B. simplicifolia* agglutinin binding) while mutant cells bound *B. simplicifolia* agglutinin, which could be eliminated by exogalactosylation. In contrast to wild-type cells, mutant cells did not bind peanut lectin (specific for terminal galactose linked to *N*-acetylgalactosamine). While mutant cells had decreased binding to (70 to 75%) *Limax flavus* agglutinin (LFA, a lectin which binds sialyl residues in a non-glycosidic linkage specific manner, see Cross et al., J. Biol. Chem., 278:4112 (2003), a copy of which is enclosed herewith) (pages 16286-7), Brandli et al. conclude that MDCKII-RCA^r cells are deficient in the addition of galactose residues to *N*- and *O*-linked glycans (page 16286).

None of the cited references discloses a cell line which has reduced levels of terminal sialic acid, e.g., reduced levels of *N*-acetylneuraminic acid (note that MDCKII-RCA^r cells bound wheat germ agglutinin which is specific for *N*-acetylglucosamine and *N*-acetylneuraminic acid). Accordingly, withdrawal of the § 102(b) rejections is respectfully traversed.

Conclusion

Applicant respectfully submits that the claims are in condition for allowance and notification to that effect is earnestly requested. The Examiner is invited to telephone Applicant's attorney at (612) 373-6959 to facilitate prosecution of this application.

If necessary, please charge any additional fees or credit overpayment to Deposit Account No. 19-0743

Respectfully submitted,

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TABLE 4. *Genetic determinants of host range or virulence of influenza virus* in vitro *and* in vivo

Virus	Host	Phenotype	Genes that specify the indicated phenotype and comments	References
A. Viruses evaluated in tissue culture of eggs:				
Avian influenza A viruses	Chicken embryo (CE) cells	Plaque formation	Cleavability of HA by CE cells	65
A/WSN/33	Human embryo fibroblasts; MDBK cells; neuroblastoma cells	Plaque formation	NA gene required T to promote cleavability of HA	402, 465, 572
A/Victoria/3/75	MDCK cells	Plaque formation	NA gene determines plaque size	395
A/FPV/Rostock/34	BHK cells	Plaque formation	PB2 determines inability to plaque	12
A/FPV/Dobson	L cells	td-hr	PB2 determines *td-hr* phenotypes	287
A/FPV/Rostock/34	MDCK cells	Plaque formation	*ts* virus (*ts* on CE cells) with PB1 or PB2 mutations cannot plaque on MDCK cells	557
A/PR/8/34	Allantoic cavity of eggs	High growth	M gene	20
B. Viruses evaluated in mammals:				
A/turkey/England/63	Mice	Hepatotropism	PA, HA, NP, M constellation required	235, 571
A/PR/8/34	Mice	Pneumovirulence	PB1 required for pneumotropism and also for enhanced replication on mouse embryo cells	626
A/England/1/61	Mice	Pneumovirulence	PB1 and PA genes both needed	170, 626
A/WSN/33	Mice	Neurovirulence	NA major determinant: M and NS contributors	85, 608
A/Okuda/57	Ferrets	Restriction of replication	HA and NA genes	91
A/Alaska/6/77	Ferrets	Infectivity	Deletion mutation affecting NS1 protein decreases replication in ferrets and MDCK cells	78
A/mallard/NY/78	Squirrel monkeys	Restriction of replication	NP gene major; M gene contributory	453
A/New Jersey/11/78	Pigs	Infectivity	Genetic dimorphism at amino acid residue 155 affects infectivity for pigs; reactivity with antisera; and yield of virus in eggs	66
Viruses evaluated in birds:				
Avian influenza A viruses	Chickens	Mortality	Viruses with HA cleavable in CE cells are pathogenic in chickens; specific sequence at HA1-HA2 junction determines cleavability	64, 65, 193
A/mallard/NY/78	Ducks	Enterotropism	HA and NA required	264
A/Udorn/307/72	Ducks	Enterotropism	Mutations at amino acid positions 226 and 228 on HA required for enterotropism	462
A/turkey/Ontario/66	Ducks	Mortality	RNA segment 3 and NP	647
A/chicken/Japan/24	Chickens	Mortality	HA major determinant; M and NA contributory	477

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Recruitment of Murine Neutrophils *in Vivo* through Endogenous Sialidase Activity*

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ABSTRACT

Upon activation with various noncytokine stimuli, polymorphonuclear leukocytes (PMNs) mobilize intracellular sialidase to the plasma membrane, where the sialidase releases sialic acid from the cell surface. This desialylation enhances PMN adherence, spreading, deformability, and motility, functions critical to diapedesis. We now have examined the role of sialidase activity in PMN adhesion to and migration across the endothelium *in vivo*. A polyclonal antibody prepared against *Clostridium perfringens* neuraminidase 1) detected surface expression of sialidase on human PMNs stimulated with IL-8 *in vitro* and on murine PMNs stimulated *in vivo*, but not on that of unstimulated cells, 2) recognized proteins in human PMN lysates and granule preparations that were not detected by preimmune antibody, 3) inhibited bacterial neuraminidase and human PMN sialidase activities *in vitro*, and 4) inhibited both pulmonary leukostasis in mice systemically infused with cobra venom factor and intrapulmonary transendothelial migration of PMNs into the bronchoalveolar

compartment of mice intranasally challenged with interleukin-8. We conclude that the chemokine interleukin-8, like other PMN agonists, induces the translocation of sialidase to the PMN surface and that surface expression of this sialidase is a prerequisite to PMN recruitment *in vivo*. The ability of antibodies raised against a prokaryotic neuraminidase to recognize eukaryotic sialidase extends the concept of the neuraminidase superfamily to mammalian enzymes. Inhibition of mobilized endogenous sialidase may provide a novel strategy for limiting the inflammatory response.

INTRODUCTION

Sialic acids are found in microbes, invertebrates and in the tissues of all mammals. Their presence on the cell surface imparts a negative surface charge and is an important determinant of that cell's interaction with other cells (*e.g.* T and B lymphocyte interaction, aggregation with similar (homotypic) or distinct (heterotypic) cell types), organic and plastic matrices, informational molecules (*e.g.* hormones and cytokines), and invading microbes (1-5). Sialic acid protects cells and molecules from immune recognition, clearance, and/or degradation (6-8), and the level of sialylation determines the rheologic properties, motility, and metastatic potential of cells (9-12).

Myeloid precursor cells in the bone marrow express abundant sialic acid. During maturation, a progressive loss of cell-associated sialic acid is associated with increases in cellular deformability and motility that permit exit from the bone marrow compartment (10). Once in the periphery, stimulation of circulating mature myeloid cells is associated with further loss of cell surface sialic acid, diminished negative surface charge, and enhanced functional activity (7). Thus, the sialic acid content of myeloid cells dynamically changes during both cellular maturation and activation.

Neuraminidases (NANases)[1] are a family of enzymes present in microbes, parasites, and mammalian tissue (where they are referred to as "sialidases"), which rapidly remove sialic acid residues, usually present at the nonreducing terminal position, from sialylated glycoconjugates (13). We observed that upon activation of polymorphonuclear leukocytes (PMNs), sialidase present within an intracellular granule compartment translocated to the plasma membrane, where 95% of the cell-associated sialic acid resides (9, 14). This sialidase translocation was accompanied by a release of cell-associated sialic acid, an increase in cellular adhesiveness, and homotypic aggregation (9). As an integral membrane protein (*i.e.* it was not released from the cell), the translocated sialidase could also desialylate adjacent cells in the local environment. Thus, during activation, PMNs can rapidly modulate surface sialic acid in an autocrine and paracrine manner. We propose that the loss of cell-associated sialic acid through the activity of mobilized sialidase may be a prerequisite event for PMN adherence to and migration across the endothelial barrier into inflamed extravascular tissues.

During acute pulmonary vascular endothelial injury and dysfunction, including the acute respiratory distress syndrome (ARDS) and immune complex-mediated lung injury, there is an early entry of PMNs into the lung microvasculature (15, 16). This PMN recruitment and activation may be in response to locally generated cytokines, such as IL-8, or to other chemoattractants, such as C5 cleavage products and

leukotriene B4 (16). IL-8, a cytokine produced by many cell types, including endothelial cells, fibroblasts, respiratory epithelial cells, macrophages, and PMNs, is a potent chemoattractant for PMNs and binds to a subfamily of related G-protein-coupled receptors (17). It is found in high concentrations in the bronchoalveolar lavage fluid of ARDS patients and in the circulation during clinical and experimental human sepsis (18, 19). In PMNs, IL-8 also increases surface expression of adhesion molecules, transendothelial migration, degranulation, and superoxide anion formation (17).

In order to exit from the circulation into an inflammatory site, PMNs must adhere to vascular endothelium, markedly alter their shape, squeeze through interendothelial cell junctions, and disengage from the undersurface of endothelial cells to enter the underlying tissue (for a review, see Ref. 20). These functions require dynamic changes in the amount of sialic acid on the PMN surface.

We previously demonstrated that a variety of PMN agonists (fMLP, phorbol 12-myristate 13-acetate, and calcium ionophore (A23187)) induce translocation of sialidase from intracellular storage sites to the plasma membrane with a release of sialic acid into the culture medium (9). Since IL-8 also activates PMNs, in this study we determined whether this endogenous chemokine also provoked surface sialic acid release. The membrane-associated sialidase from human PMNs has been neither cloned nor purified. We therefore tested the hypothesis that the previously described superfamily of prokaryotic NANases (13) might extend to human PMN sialidase and that antibodies raised against one bacterial NANase (*Clostridium perfringens*) may cross-react with conserved regions of NANase or sialidase obtained from other species (human or murine PMN sialidase). In this study, we demonstrate that sialidase inhibition *in vivo* can profoundly reduce PMN influx into inflammatory sites in three animal models of PMN recruitment.

EXPERIMENTAL PROCEDURES

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Preparation of Polyclonal Anti-NANase Antibody-- New Zealand White rabbits were immunized with 50 μg of *C. perfringens* NANase, type V (Sigma) The initial intravenous injection was administered in Freund's complete adjuvant, and a booster dose of 50 μg was given intramuscularly on day 14 in Freund's incomplete adjuvant. Boosts of 50 μg of NANase were given subcutaneously without any adjuvant at 4, 10, 16, and 22 weeks. At 1 week after the last boost, serum was obtained, and the IgG was purified by affinity chromatography on a protein G column (Amersham Biosciences), eluted with glycine-HCl buffer (0.15 M, pH 2.5), and immediately neutralized with 0.15 M Tris to a pH of 6.8-7.2. The combined fractions were filter-sterilized (0.2 μM), and aliquots of the antibody preparation were stored at −20 °C.

Preparation of Human PMN Lysates and Granules-- Human PMNs were stimulated for 10 min at 37 °C with phorbol 12-myristate 13-acetate (100 ng/ml) and centrifuged, and the pellets were treated with 1% Nonidet P-40 and DNase I (final concentration of 10 μg/ml). The PMN lysates were then subjected to three cycles of freeze/thawing in methanol and dry ice. For preparation of sialidase in human neutrophil granules, a human volunteer was treated with recombinant granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA) (5 μg/kg at 48 and 24 h prior to pheresis) under a protocol approved by

the University of Maryland at Baltimore Institutional Review Board, as previously described (21). Following leukapheresis, cells were separated with Histopaque, and erythrocytes were lysed by hypotonic lysis. After treatment with diisopropyl fluorophosphate to inhibit proteolysis, cells were suspended in relaxation buffer (100 mM KCl, 10 mM PIPES, pH 7.4, 3 mM $MgCl_2$, 3.5 mM NaCl), to which 10 μM phenylmethylsulfonyl fluoride, 1 mM ATP, and 1 mM EGTA were added. PMNs were subjected to nitrogen cavitation (40 p.s.i. for 15 min), and the cavitate was centrifuged at 500 × *g* for 5 min to remove unbroken cells and nuclei. The supernatant was then centrifuged at 10,000 × *g* for 20 min, and the granule-enriched pellet was resuspended in relaxation buffer and solubilized at 4 °C with 0.4% deoxycholate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 8.0, for 1 h. The insoluble material was centrifuged at 37,000 × *g* for 1 h at 4 °C, and the supernatant was dialyzed extensively against 25 M NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 8.0, at 4 °C. The protein concentration was determined by a modified Lowry assay (22).

Western Blot Analysis-- Solubilized protein samples (50 μg) were boiled in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 0.6 mM β-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol and separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride and nitrocellulose membranes (23). Proteins immobilized to polyvinylidene difluoride were stained with Coomassie Blue. After electrotransfer, Western blot analysis using anti-NANase antibodies was performed on the nitrocellulose membrane, which had been blocked overnight at 4 °C in 0.5% bovine serum albumin, 0.05% Tween 20 in PBS, pH 7.4 (PBT buffer). Sialidase activity was detected by incubating the membrane with 5 ml of PBT containing polyclonal IgG antibodies raised against clostridial NANase (dilution 1:50) for 1 h at room temperature and then washed twice (10 min each) with 5 ml of PBT. Bound anti-sialidase antibodies were detected by incubating the membrane with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted at 1:5000 in PBT for 45 min at room temperature. The membrane was washed twice as described above, and bound peroxidase activity was detected with 3,3'-diaminobenzidine in the presence of $CaCl_2$ as described (24).

Immunoprecipitation of PMN Sialidase-- Fresh human PMNs were lysed with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, 1 mM vanadate, 1 mM sodium fluoride, 10 mM disodium pyrophosphate, 500 μM paranitrophenol, and 1 mM phenyl arsine oxide (all purchased from Sigma). The PMN lysates were precleared by incubation with goat anti-rabbit IgG cross-linked to agarose (Sigma) for 1 h at 4 °C and then incubated overnight with rabbit antibody raised against *C. perfringens* neuraminidase or preimmune rabbit serum. The resultant immune complexes were immobilized by incubation with goat anti-rabbit IgG cross-linked to agarose for 2 h at 4 °C, centrifuged, washed, boiled for 5 min in sample buffer, and again centrifuged. The supernatants were then processed for immunoblotting with the same anti-neuraminidase antibody as described above. The blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Transduction Laboratories) and developed with ECL.

Detection of Sialidase on the PMN Surface-- PMNs were prepared from heparinized blood drawn from healthy volunteers as described (9) or by cardiac puncture of mice. The ability of the polyclonal anti-

NANase antibody to bind to surface sialidase on intact PMNs harvested from mice 2 h after intraperitoneal injection of recombinant human IL-8 (Calbiochem) and on human PMNs stimulated *in vitro* with either IL-8 or fMLP in the presence of cytochalasin B was determined by flow cytometry (Facscan II, Becton-Dickinson, Mountain View, CA) and analyzed with Lysis II software as described (6). Normal rabbit IgG (Sigma) or rabbit preimmune IgG was used as the irrelevant controls.

Detection of Changes in Cell Surface Sialic Acid with Two Distinct Lectins-- Heparinized peripheral blood from healthy volunteers was treated with 0.83% ammonium chloride (Sigma) for 10 min at 21 °C to remove erythrocytes. Leukocytes were pelleted, washed at 4 °C in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS^-) (Invitrogen) supplemented with 0.2% heat-inactivated fetal calf serum (Hyclone, Logan, UT), and repelleted. The leukocytes were resuspended to a concentration of 5×10^6 cells/ml in PBS^- with 0.2% heat-inactivated fetal calf serum and were exposed to IL-8, NANase, or medium alone. After treatment, cells were washed and labeled with fluorescein isothiocyanate (FITC)-labeled *Limax flavus* lectin (EY Laboratories, San Mateo, CA), which binds to sialyl residues in a non-glycosidic linkage-specific manner or with the lectin from *Arachis hypogaea* (peanut agglutinin lectin (PNA), EY Laboratories), which binds to terminal β-galactose residues on glycoconjugates (usually after removal of terminal sialyl residues). Samples of surface lectin-labeled cells were then acquired and analyzed on a FACScalibur flow cytometer equipped with Cell Quest software (Becton Dickinson, San Jose, CA). Based on forward and right angle light scatter properties (linear scale), an electronic gate was placed around the PMNs exclusively. The measurement of green fluorescence (MPFL) (log scale) was obtained from unstained, nonstimulated, and NANase- or IL-8-treated PMNs.

High Performance Anion Exchange Chromatography Coupled with Pulsed Electrochemical Detector Analysis of Sialyl Residues-- A Dionex DX600 chromatography system (Dionex Corp., Sunnyvale, CA) equipped with an electrochemical detector (ED50; Dionex Corp.) was used. Cell surface sialic acids were released by the treatment of human PMN suspensions (25×10^6 PMNs in 250 μl) with either medium alone for 15 min, NANase (30 milliunits/ml) for 60 min, IL-8 (150 ng/ml) for 15 min, or fMLP (10^{-6} M) for 5 min, all at 37 °C. Supernatants were obtained for analysis of sialyl residues, and cell pellets were resuspended to their original volume in culture medium, and each of the four PMN preparations was then treated with NANase (30 milliunits/ml for 60 min) to further release sialyl residues not released by the initial treatments. The sialic acid content of the two supernatants was analyzed with a CarboPac-PA1 column (4 × 250 mm), with 0.1 M sodium hydroxide and 1 M sodium acetate in 0.1 M NaOH as eluents. A gradient of 5-20% over 0-15 min was run at a flow rate of 1 ml/min. Under this condition, *N*-acetylneuraminic acid was eluted at 9.3 min. Sialic acid samples were quantified by integration of the peak area using a standard *N*-acetylneuraminic acid solution as the reference, with an area of 6.8 corresponding to 1 nM sialic acid.

Measurement of NANase Activity-- The sialidase activity was detected in PMN extracts (10^7 PMN/ml) using an established thiobarbituric acid assay to measure sialic acid release from an artificial substrate, neuraminyllactose (Sigma) (25). Human PMNs (10^7 PMN/ml) were stimulated with IL-8 (0.1-10 ng/ml) for 1 h at 37 °C and lysed, and the lysates were assayed for sialidase activity. A standard curve of NANase activity was obtained by testing increasing concentrations of *C. perfringens* NANase, type V

(1-25 milliunits/ml) in the presence of a fixed concentration (1 mg/ml) of neuraminyllactose in 0.8 M sodium acetate, pH 5.5, and incubated for 2 h at 37 °C. Alternatively, *C. perfringens* NANase (2 milliunits) or PMN lysate (100 μg of protein) was incubated for 1 h at 37 °C with 0.7 mM PNP-NANA (Sigma) as a substrate in 0.8 M sodium acetate, pH 4.6, in a final volume of 200 μl. The reaction was stopped by adding 1 ml of 0.085 M glycine-carbonate, pH 10, and the nitrophenol released was measured at A_{405} with *p*-nitrophenol used as a standard, as previously described (26).

Effect of Anti-NANase-IgG on Cobra Venom Factor (CVF)-induced Pulmonary Leukostasis-- The systemic infusion of CVF activates complement, generates the C5 cleavage product, C5a, and induces pulmonary leukostasis (27). To assess the effect of sialidase neutralization on CVF-mediated leukostasis, we pretreated 6-8-week-old ICR mice (Charles River, Wilmington, MA) with either nonimmune rabbit IgG or anti-NANase-IgG. After 1 h, CVF (Quidel, San Diego, CA) or PBS was injected intravenously in a total volume of 0.1 ml. PMN recruitment into the lung was determined by both biochemical and histologic techniques. The right lung was used to quantify myeloperoxidase (MPO) activity/0.1 g of tissue (28) (see below), whereas the left lung was processed for histology with hematoxylin/eosin staining (29).

Tissue MPO Assay-- MPO is a heme-containing enzyme found in the azurophilic granules of PMNs and has been successfully used as a biochemical marker of intrapulmonary PMNs (27). Quantification of the number of PMN per 0.1 g of tissue was done according to the method of Suzuki *et al.* (28) on lung homogenates. The MPO activity was interpolated from a standard curve using purified human MPO (Sigma) and correlated with the MPO activity of murine PMNs prepared by Ficoll-Hypaque density gradient centrifugation (380 *g*, 20 °C, 40 min) of blood obtained by cardiac puncture.

Preliminary experiments confirmed earlier observations that CVF-induced pulmonary leukostasis was maximal at 0.5 h after injection and then abated (27). At 0.5 h, mice were sacrificed and bled, and MPO activity in the nonperfused lung homogenates was measured. In preliminary experiments, we observed that perfusing the lungs in this model removed all PMNs within the blood vessels and thus abolished any effects of CVF on pulmonary leukostasis (data not shown). Accordingly, in these experiments lung MPO was determined on unperfused lungs.

Effect of Anti-NANase IgG on IL-8-induced PMN Recruitment into the Bronchoalveolar Compartment-- Since IL-8 is detected in the lungs after bacterial infection (30) and during ARDS (16), we developed an animal model to assess PMN extravasation from the pulmonary vasculature into the interstitium and alveolar space in response to this chemokine. Following anesthesia with ketamine hydrochloride (80 mg/kg) and xylazine (8 mg/kg) given intraperitoneally (29), ICR mice were administered intravenously either anti-NANase IgG or nonimmune rabbit IgG (Sigma). After 1 h, the mice were given equivalent volumes (50 μl) of either nonpyrogenic normal saline (see Fig. 3, *NS*) or rhu IL-8 (2.5 μg/mouse) into the left nasal opening. PMN recruitment into the lung was followed over a 4-h period, since preliminary experiments indicated that 4 h was the time of maximal PMN influx. To exclude intravascular PMN, lungs were perfused with 5 ml of phosphate-buffered saline containing 1 unit/ml of heparin via the right ventricle.

Effect of Anti-NANase IgG on Circulating and Tissue PMN Levels-- To determine whether anti-NANase

antibodies that bound to murine PMNs *in vitro* resulted in accelerated clearance of PMNs from the circulation, we measured the level of circulating PMNs in mice 1.5 and 5 h after intravenous administration of either normal rabbit IgG or rabbit anti-NANase antibody. The number of PMNs was manually counted in a hematocytometer. At 5 h, tissue leukostasis in the liver and spleen was measured by the MPO assay.

Mouse Peritonitis Model-- Since the mechanism(s) of PMN extravasation from the systemic and pulmonary circulation appear to be distinct (3, 24), we also determined whether sialidase also might be required for optimal PMN recruitment into the peritoneal cavity. Mice were given rhu IL-8 intraperitoneally, and the PMN influx was followed at various time points by direct counts of PMNs harvested by peritoneal lavage. In preliminary experiments, the PMN influx was optimal 4 h after IL-8 challenge. In this model, we utilized soluble sialic acid (Sigma) (31) as a competitive inhibitor of sialidase administered either as a single bolus prior to the IL-8 challenge or in multiple doses before and after rhu IL-8 administration. In control animals, equivalent volumes (0.1 ml) of pyrogen-free, normal saline were administered at the same time points in lieu of sialic acid.

Statistical Analysis-- Comparisons of the -fold increase were done with the two-tailed Mann-Whitney *U* test. All data are expressed as median ± quartiles.

RESULTS

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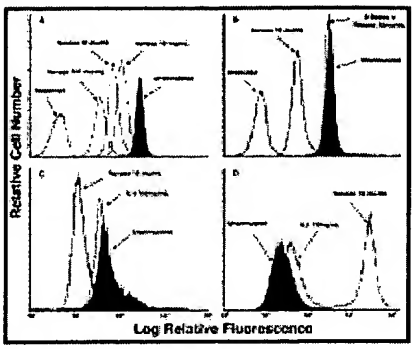
Effect of IL-8 on PMN Release of Cell-associated Sialic Acid-- We have demonstrated that PMN sialidase resides with a granule subpopulation (9). Since IL-8 not only attracts but also stimulates PMN exocytosis (17), we determined whether IL-8 also provoked surface sialic acid release. Sialic acid release from the surface of human PMNs could be demonstrated by a decrease in the amount of the sialic acid-binding lectin, *L. flavus*, and an increase in PNA lectin binding on the cell surface of IL-8 treated nonpermeabilized cells (Fig. 1). Increasing concentrations of bacterial neuraminidase induced a dose-dependent loss of lectin binding to the PMN surface (Fig. 1*A*). This loss of surface fluorescence from PMNs treated with one concentration of neuraminidase was reversed by the competitive sialidase inhibitor, 2-deoxy-NANA (Fig. 1*B*). IL-8 decreased lectin binding to PMNs compared with untreated PMN controls (Fig. 1*C*). In contrast to the *L. flavus* lectin, the PNA lectin binds to desialylated glycoproteins that have a terminal β-galactose residue. Consequently, instead of the decreased fluorescence observed with the sialic acid-binding lectin following NANase treatment or IL-8 mobilization of an endogenous sialidase (Fig. 1*C*), we observed an increased binding of FITC-labeled PNA following these same desialylating treatments (Fig. 1*D*).

Fig. 1. IL-8 decreases binding of the sialic acid-binding lectin, *L. flavus*, to the surface of human PMNs. PMNs were isolated from the peripheral blood of healthy human donors. Following treatment with NANase, IL-8, or medium alone (unstimulated), PMNs were stained with FITC-labeled *L. flavus* lectin (*A-C*) or FITC-labeled *A. hypogaea* (PNA)



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lectin and analyzed by flow cytometry. *A*, a progressive loss of surface fluorescence was observed following treatment with increasing doses (10-100 milliunits/ml) of NANase; *B*, the addition of the competitive NANase inhibitor, 2-deoxy-NANA, reversed the loss of fluorescence that occurred with the NANase treatment. No similar reversal was observed when NANase was co-incubated with 2,3-keto-octonic acid (*KDO*) a molecule with similar size and charge as 2-deoxy-NANA, but without NANase inhibitory activity (data not shown); *C*, treatment of PMNs with either IL-8 (150 ng/ml) or NANase caused a loss of fluorescence from the surface of PMNs compared with unstimulated cells. *D*, the loss of sialic acid from the surface of PMNs also could be demonstrated by an increase in PNA-binding activity following treatment with either NANase or IL-8. In contrast to the *L. flavus* lectin, the PNA lectin reacts with desialylated glycoconjugates. Each cytometric analysis is representative of at least two experiments.

Sialic acid release from the surface of PMNs could also be demonstrated by HPLC. Following exposure of 25×10^6 human PMNs to IL-8, fMLP, or medium alone, the amounts of sialyl residues released from PMNs into the supernatant were below the level of detection of the Dionex DX600 chromatography system, a highly sensitive detection system for sialic acid. Only after NANase treatment was the release of sialyl residues consistently detectable. As an alternative strategy to quantify PMN desialylation, we therefore measured how much NANase-releasable sialyl residues remained on the PMN surface after treatment with agonists. Following treatment with IL-8 or fMLP or exposure to medium, the pellets of each of the three PMN preparations were then subjected to NANase treatment. The amount of sialic acid released into the supernatant by NANase treatment of cells initially incubated in medium alone was defined as the total NANase-releasable sialic acid. After treatment with IL-8 and fMLP, there were decreases in cell surface-associated sialyl residues of 14 ± 5 and $10 \pm 1\%$, respectively ($p = 0.06$ and <0.01, respectively, compared with PMNs exposed to medium alone, by one-tailed, unpaired t test). Thus, as described for exogenous PMN agonists (9), IL-8 induced PMN desialylation. Therefore, IL-8-induced PMN desialylation could be demonstrated by both HPLC and flow cytometry with two distinct lectins. This loss of PMN surface sialic acid residues following activation with an endogenous mediator, IL-8, was compatible with translocation of sialidase activity to the plasma membrane with attendant surface desialylation.

Ability of Anti-NANase IgG to Recognize Human PMN Sialidase-- To detect the mobilization of human PMN sialidase, we prepared antibodies to clostridial NANase for use in both Western blot analysis of PMN lysates and granule preparations and in analysis of intact cells by flow cytometry. Coomassie Blue staining of proteins from clostridial NANase revealed a major band with an apparent M_r of 91,000 (Fig. 2, *lane 2*). Immunoblotting with the polyclonal anti-NANase IgG antibody revealed multiple PMN lysate (*lane 4*) and granule (*lane 8*) proteins as well as a strong reaction with bacterial NANase protein (*lane 3*). In contrast, preimmune IgG did not react with either preparation (*lanes 5* and *9*). Since we failed to detect a band in the PMN lysate that co-migrated with the immunoreactive bands in the granule

preparation (*lane 8*), we used immunoprecipitation with anti-NANase antibody to enrich for and detect a cross-reactive protein in the PMN lysate (*lane 6*). Nonimmune IgG did not detect these bands (*lane 7*) by immunoprecipitation. The anti-NANase antibody neither recognized human purified MPO (Sigma) on Western blot nor interfered with its enzymatic activity *in vitro* (data not shown). Conversely, human purified MPO did not block the ability of anti-NANase antibody to recognize NANase on Western blot analysis, whereas the addition of clostridial NANase to the same antibody preparation decreased NANase immunoreactive signal (data not shown). Thus, our antibody raised against a bacterial NANase recognized epitopes on human proteins that reside within PMN granules, whereas it did not react with a second protein that is known to reside within the same PMN granule population.

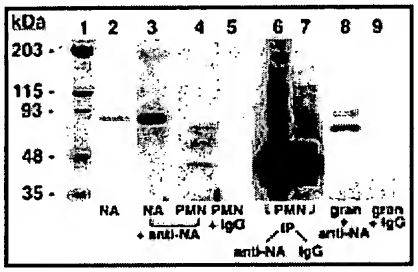

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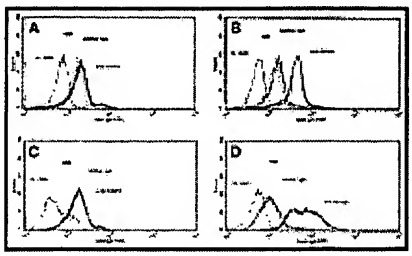
Fig. 2. Immunoprecipitation and immunoblotting of human PMN lysates (*PMN*) and granules (*gran*) with antibodies raised against clostridial neuraminidase (*NA*). Solubilized PMN proteins were resolved by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride. Coomassie Blue stain was used to detect NANase (*lane 2*). Putative NANase/sialidase was detected by incubating the transfers with polyclonal IgG antibodies against clostridial neuraminidase (*anti-NA*) or preimmune IgG (*IgG*). Bound antibodies were detected by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG and developed with enhanced chemiluminescence. Each *lane* was loaded with molecular mass markers (*lane 1*), clostridial NANase (0.1 μg) (*lanes 2* and *3*), PMN lysate (50 μg) (*lanes 4* and *5*), and PMN granules (50 μg) (*lanes 8* and *9*). To enrich for and detect a cross-reactive PMN protein with a gel mobility comparable with those detected in granules and NANase, we performed immunoprecipitation with anti-NANase (*lane 6*) and nonimmune (*lane 7*) antibodies. The heavy chains of the immune and nonimmune antibodies are evident in both lanes. The position of the molecular mass standards (kDa) are shown on the *left*. Each blot is representative of three separate experiments.

Binding of Anti-NANase to Intact Murine and Human PMNs-- The rabbit anti-NANase antibody also was used with flow cytometry to document the presence or absence of translocated enzyme on the surface of resting and IL-8-activated PMNs isolated both from humans and mice. Human PMNs were stimulated *in vitro*, and murine PMNs were stimulated *in vivo* with IL-8. Incubation of unstimulated human PMNs with normal rabbit IgG and anti-NANase IgG each yielded similar binding intensities (Fig. 3*A*). In contrast, IL-8 increased anti-NANase IgG binding to the PMN surface (Fig. 3*B*), whereas normal rabbit IgG binding was unchanged. Normal rabbit IgG binding to stimulated and nonstimulated PMNs were no different than that observed with nonspecific binding (*i.e.* binding of secondary antibody in the absence of primary antibody).

Fig. 3. Flow cytometric analysis of human (*A* and *B*) and murine (*C* and *D*) PMNs treated *in vitro* or *in vivo* with



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either normal saline or IL-8. Human PMNs were treated with medium alone (*A*) or rhu IL-8 (250 ng/ml) (*B*) for 45 min at 37 °C. Murine PMNs were purified from blood 2 h after intraperitoneal administration of either normal saline (*NS*) (*C*) or IL-8 (750 ng) (*D*). PMNs were incubated with nonimmune rabbit IgG (Sigma) or polyclonal rabbit anti-NANase IgG followed by FITC-labeled goat anti-rabbit IgG as the secondary antibody. Nonspecific binding (*NSB*) of fluorescently labeled secondary antibody was determined in the absence of cell treatment with anti-NANase or normal rabbit IgG. An increase in the intensity of binding of anti-NANase antibodies to activated PMNs was examined by a shift in mean channel fluorescence.

To determine whether anti-NANase antibody could bind to PMNs stimulated *in vivo*, mice were treated with either IL-8 or PBS 2 h prior to cardiac puncture, when PMNs were harvested and studied for anti-NANase IgG and control IgG binding. There was no difference between the binding of nonimmune rabbit IgG and anti-NANase IgG to PMN from saline-injected mice (Fig. 3*C*). After IL-8 challenge, PMN surface expression of an epitope was recognized with anti-NANase antibody increased (Fig. 3*D*). These data indicate that a clostridial NANase cross-reactive antigen is expressed on the surface of PMNs only upon activation both *in vitro* and *in vivo*. Thus, areas of homology in the neuraminidase/sialidase superfamily exist such that antibodies against clostridial NANase also recognized epitopes in an endogenous sialidase of PMNs isolated from both mice and humans.

Inhibition of Clostridial NANase and PMN Sialidase Activities by Anti-NANase Antibody-- The anti-NANase antibody recognized both clostridial NANase and human PMN proteins (Figs. 2 and 3). To establish whether this same antibody could inhibit NANase/sialidase catalytic activity *in vitro*, clostridial NANase (25 milliunits/ml) and sialidase-containing PMN lysates were incubated for 1.5 h at room temperature with different concentrations of anti-NANase IgG or preimmune IgG, followed by the addition of these enzyme/antibody mixtures to the artificial NANase substrate, neuraminyllactose, for 1 h at 37 °C, and liberated sialic acid was measured (9) (Table I). At an IgG antibody concentration of 10 μg/ml, there was 56% inhibition of bacterial neuraminidase activity compared with no inhibition with preimmune IgG. Whereas the anti-NANase IgG inhibited sialidase activity in PMN lysates in a dose-related manner unlike preimmune IgG, it was unable to achieve 50% inhibition, even with the addition of higher anti-NANase IgG levels.

Table I

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Anti-C. perfringens neuraminidase antibodies inhibit both C. perfringens neuraminidase and human neutrophil sialidase activities

Neutrophils were isolated from the peripheral blood of healthy human volunteers, adjusted to a concentration of 1-2 × 10^8 cells/ml in Tris-buffered saline, pH 7.2, that also contained 0.5% Triton X-100 and complete protease inhibitor mixture tablet (Roche Molecular Biochemicals), and lysates were prepared by disruption with three cycles of freeze/thawing in ethanol/dry ice. After the mixture was vigorously vortexed, supernatants were obtained following centrifugation (150,000 rpm at 4 °C for 5 min in a

microcentrifuge). This neutrophil lysate or *C. perfringens* neuraminidase was incubated with different concentrations of either pre- or postimmune rabbit polyclonal anti-neuraminidase IgG that was eluted from a protein G column (Pierce). After 90 min of incubation at room temperature, the antibody and enzyme source was added to the artificial substrate, neuraminyllactose (200 μg/ml) and incubated for 1 h at 37 °C. A modification of the thiobarbituric acid assay was then performed in a 96-well microtiter plate, and absorbance was read at A_{540} in a microplate reader (Molecular Devices, Palo Alto, CA). The data are from three separate experiments each performed in duplicate and are expressed as mean ± S.E. μg of sialic acid liberated from the artificial substrate. A standard curve of sialic acid (*N*-acetylneuraminic acid; Sigma) was performed in each experiment. The amount of sialic acid liberated by PMN lysates in each experiment was standardized to the total protein concentration of each PMN lysate and expressed as μg/mg of protein.

Anti-NANase Antibodies Inhibited CVF-induced Pulmonary Leukostasis-- Since the polyclonal anti-NANase antibodies inhibited both clostridial NANase and PMN sialidase activities *in vitro*, we examined whether the same antibody preparation exerted any inhibitory activity *in vivo*. To determine whether anti-NANase antibodies were effective in blocking an early step in non-IL-8-induced intrapulmonary recruitment of PMNs (*i.e.* PMN adhesion to the lung microvascular endothelium), mice were infused with CVF intravenously, and pulmonary leukostasis was quantified (27). In control mice, a mean ± S.D. of $6.6 \pm 0.72 \times 10^6$ PMN/0.1 g of nonperfused lung tissue ($n = 4$) was found. With CVF treatment, there was a 4-5-fold increase in PMNs to $32 \pm 19.9 \times 10^6$ PMN/0.1 g of lung tissue at 0.5 h, the time of peak PMN recruitment established in an earlier study (27) and confirmed in preliminary studies (data not shown). Anti-NANase antibody reduced CVF-induced pulmonary leukostasis compared with treatment with nonimmune IgG (Fig. 4*A*). Microscopic examination of lung sections from CVF-infused mice revealed PMNs within alveolar septal wall capillaries but not within alveoli (Fig. 4*B*). Pretreatment with anti-NANase IgG markedly reduced the intravascular PMNs (Fig. 4*C*), whereas normal rabbit IgG did not (Fig. 4*B*). These data are compatible with our hypothesis that endogenous PMN sialidase activity is required for increased PMN adhesion to the endothelial surface in CVF-induced pulmonary leukostasis. Interestingly, when examined at 4 h, a time at which the CVF-induced leukostasis was no longer evident, there was no difference in the PMN level in the lungs of mice treated with immune or nonimmune IgG. This suggests that in the absence of PMN activation and surface expression of sialidase, the enzyme was no longer accessible to the anti-NANase antibody.

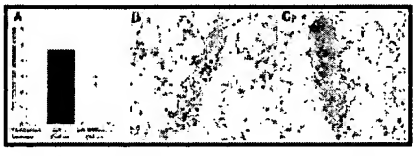

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Fig. 4. Anti-NANase antibody reduced pulmonary leukostasis in CVF-treated mice. At 1 h after intravenous (*i.v.*) pretreatment with either nonimmune or anti-NANase IgG (20 μg/g), mice were infused with CVF (25 units/kg) intravenously (*A*). MPO activity was measured in nonperfused lungs as a biochemical marker of intrapulmonary PMNs. Saline-infused mice had no increase in lung MPO activity compared with untreated mice (data not shown). Lung sections were obtained from mice pretreated with either nonimmune IgG (*B*, hematoxylin/eosin (*H&E*) stain, ×560 magnification) or with anti-NANase IgG (*C*,

hematoxylin/eosin stain, ×560 magnification). The *arrows* point to intravascular PMNs.

Effect of Anti-NANase IgG on IL-8-induced Recruitment of PMNs into the Brochoalveolar Compartment-- Since immunoblockade of endogenous sialidase activity diminished PMN-to-endothelial adhesion in response to systemic complement activation, we asked whether this same intervention might restrain paracellular movement of PMNs across the endothelial barrier in response to an extravascular chemotactic gradient. We have previously demonstrated that both sialic acid and 2-deoxy-NANA, competitive inhibitors of NANase, inhibit sialidase activity of human PMN *in vitro* (9). To extend these findings to our *in vivo* system, we studied whether the anti-NANase IgG, which recognized sialidase on the surface of activated murine and human PMNs and inhibited catalytic activity *in vitro*, also was capable of modifying *in vivo* PMN behavior mediated through desialylation. In preliminary experiments, intranasal administration of IL-8 induced a time-dependent increase in MPO activity in homogenates of perfused lungs for up to 4 h (data not shown). This increase in lung MPO coincided with PMN migration across the pulmonary vascular endothelial barrier into alveoli detected by light microscopy.

The mean ± S.D. base-line PMN content in perfused lungs of mice was $9.3 \pm 0.4 \times 10^5$ cells/0.1 g of tissue ($n = 4$). At 4 h after intranasal saline administration, lung PMN content increased 2-fold over base line; IL-8 administration induced >4-fold increase (Fig. 5*A*). Pretreatment with anti-NANase antibody intravenously decreased the IL-8-induced recruitment of PMNs to the lungs >2-fold, compared with pretreatment with nonimmune IgG. Histologic examination of the lungs following IL-8 administration revealed intra-alveolar PMNs (Fig. 5, *B* and *C*). This mouse model involving an intranasal challenge with IL-8 is therefore an *in vivo* model of transendothelial PMN migration across the pulmonary alveolar-capillary barrier. The decrease in MPO activity in the anti-NANase IgG-treated lungs reflected the lower number of intra-alveolar PMNs on histologic examination of lung sections (Fig. 5, compare *B* and *D*). Therefore, not only is endogenous sialidase activity required for sequestration of PMNs within the pulmonary microvasculature; it is also a prerequisite for their mobilization into the alveolar space.

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Fig. 5. Anti-NANase antibodies reduce PMN transendothelial migration into the alveoli of IL-8-treated mice. Mice pretreated intravenously (*i.v.*) with either nonimmune IgG or anti-NANase IgG (each given at 20 μg/g, body weight) were administered either normal saline or IL-8 (2.5 μg/mouse) in 50 μl intranasally (*i.n.*) 1 h later. *A*, MPO activity was measured at 4 h as a biochemical marker of PMNs. The -fold increase over base-line levels was calculated by dividing the number of PMN/0.1 g of tissue of each animal by the base-line value for the particular treatment. *B* and *C*, lung sections were obtained from IL-8-challenged mice following pretreatment with either nonimmune IgG (*B*, hematoxylin/eosin (*H&E*) stain, ×560 magnification; *C*, hematoxylin/eosin stain, ×900 magnification) or anti-NANase IgG (*D*, ×560 magnification). The *arrows* indicate intra-alveolar PMNs.

Effect of Anti-NANase Antibody on Levels of Circulating and Intrapulmonary PMNs-- Intravenous infusion of anti-NANase antibody to mice did not decrease circulating PMNs. At 1.5 h after intravenous administration of anti-NANase antibody, there was a mean ± S.D. of $1.9 \pm 1.1 \times 10^6$ PMN/ml of blood ($n = 5$) compared with a preinfusion level of $1.8 \pm 0.8 \times 10^6$ PMN/ml, and at 5 h there were $2.7 \pm 1.1 \times 10^6$ PMN/ml. At 5 h, no differences in either liver or spleen MPO could be demonstrated between mice receiving anti-NANase antibody and those receiving normal rabbit IgG ($0.53 \pm 0.1 \times 10^6$ PMN/0.1 g of tissue *versus* $0.8 \pm 0.54 \times 10^6$ PMN/0.1 g of tissue in the liver ($n = 5$) and $17.5 \pm 5.3 \times 10^6$ PMN/0.1 g of tissue *versus* $19.8 \pm 6.3 \times 10^6$ PMN/0.1 g of tissue in the spleen ($n = 5$)).

Systemic Infusion of a Competitive Sialidase Inhibitor, Sialic Acid, Decreases Intraperitoneal Recruitment of PMNs-- Intraperitoneal administration of IL-8 increased peritoneal total leukocytes (2.3-fold) and, more specifically, PMNs (6-fold) at 4 h compared with the saline-treated controls (Table II). A single dose of sialic acid, a competitive inhibitor of sialidase activity (31), 5 min prior to IL-8 reduced the intraperitoneal recruitment of total leukocytes and PMNs, but these reductions did not achieve statistical significance compared with those seen with saline administration before IL-8 treatment. In contrast, following multiple doses of sialic acid, intraperitoneal total leukocytes and PMNs were both significantly decreased. These combined data suggest that the sustained presence of sialic acid within the circulation effectively reduced recruitment of leukocytes, especially PMNs, into an inflammatory site. Thus, immunologic and pharmacologic interventions that target PMN surface sialidase diminished PMN recruitment to more than one relevant body compartment.

View this table: [in this window] [in a new window]	**Table II** **A competitive inhibitor of sialidase, sialic acid, inhibits IL-8-induced influx of PMN into the mouse peritoneum**

DISCUSSION

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These studies have demonstrated that targeting endogenous PMN sialidase activity through either immunoblockade or competitive inhibition altered PMN trafficking to an inflamed site. These interventions decreased PMN recruitment in response to two distinct inflammatory stimuli: a chemokine (IL-8) or CVF-generated complement cleavage products. The ability of IL-8 to mobilize sialidase to the PMN surface (Fig. 3) and to increase the release of cell-associated sialic acid (Fig. 2) was comparable with what we have reported for exogenous, nonphysiological agonists (9). Whereas some desialylating stimuli are not chemoattractants (*e.g.* phorbol 12-myristate 13-acetate and calcium ionophore), the ability of the chemokine, IL-8, to augment transendothelial migration of PMNs *in vivo* may be related in part to its sialidase-mobilizing activity. Thus, these studies extend the concept of PMN activation-induced translocation of sialidase and surface desialylation in response to endogenous stimuli (IL-8) and suggest that this response may be central to PMN recruitment *in vivo*.

A novel finding in this study was the ability of antibody elicited against a bacterial NANase to recognize mammalian sialidase(s) in both human and murine PMNs by both Western analysis and flow cytometry (Figs. 2 and 3). With intact nonpermeable cells, the sialidase was accessible only on the surface of activated PMNs *in vitro* and *in vivo*. The same anti-clostridial NANase antibody that recognized and bound sialidase on the surface of intact PMNs as well as in PMN lysates and granule preparations and that neutralized the catalytic activity of both the bacterial NANase and PMN sialidase *in vitro* also altered the trafficking of murine PMNs *in vivo*. The polyclonal antibody selectively targeted sialidase, since it failed to recognize or inhibit the activity of another PMN granular resident enzyme, MPO. That polyclonal antibody preparations from multiple rabbits exhibited similar binding and functional properties with regard to human and murine PMN sialidase activity suggests that the anti-clostridial NANase antibodies recognize mammalian sialidase. These studies therefore extend the concept of a prokaryotic NANase (sialidase) superfamily (13) to the eukaryotic sialidases that cleave sialyl residues from PMN surface glycoconjugates.

The anti-clostridial NANase antibody was active in animal models of PMN recruitment to both intrapulmonary and extrapulmonary sites. CVF-generated complement cleavage products and IL-8 each evoked different types of PMN behavior within the lungs (pulmonary intravascular leukostasis by systemic complement activation *versus* transendothelial migration of PMN into alveoli in response to an IL-8 gradient), with distinct kinetic profiles (peak PMN recruitment at 0.5 and 4.0 h, respectively). Pretreatment of mice with anti-NANase antibodies prevented pulmonary leukostasis (*i.e.* PMN adhesion in the absence of migration). This is an early and rapid event that may occur following systemic complement activation such as demonstrated here experimentally with cobra venom factor (Fig. 4) or clinically following hemodialysis (32). This antibody also reduced extravasation of PMNs into the alveoli of IL-8-treated lungs. Whereas this model may also require PMN adhesion, it also demands that PMNs become highly deformed and motile as they squeeze through the interendothelial junction in response to a local chemotactic gradient.

The recruitment of PMNs to an extrapulmonary inflammatory site was inhibited by competitive pseudosubstrate inhibition of neuraminidase/sialidase activity (Table II). Thus, PMN recruitment to both pulmonary and extrapulmonary inflammatory sites by two distinct stimuli could be inhibited by either immunologic or pharmacologic blockade.

Sialidases differ in the type of sialyl glycosidic linkages they cleave as well as in their tissue and subcellular distributions (26, 33-35). Many microbial sialidases have been cloned and sequenced (13). Mammalian sialidase has been cloned from the cytosol of rat muscle (35) and Chinese hamster ovary cells (33); however, a role for these gene products in the immune response has not been proposed. Recently, a nonlysosome-associated human sialidase was cloned from the major histocompatibility complex region of human Epstein-Barr virus-infected lymphoblastoid cells (26). The molecular mass of this enzyme (16 kDa), believed to be similar to human lymphocyte sialidase, is different from the apparent molecular masses of the multiple protein bands in PMNs detected by the polyclonal anti-NANase antibody (26). Sialidase is present in lymphocyte lysosomes as a multienzyme complex that translocates to the surface upon cell activation. Phosphorylation of an internalization signal on the translocated sialidase prevents its endocytosis (36). Anti-NANase IgG appears to recognize the larger of

two sialidase isoforms produced by *C. perfringens* (37).

IL-8 treatment of PMNs resulted in decreased cell-associated sialic acid detected by HPLC and the loss of binding of a sialic acid-binding lectin to the cell surface with a concomitant increase in binding of a lectin (PNA) known to react strongly with desialylated glycoconjugates (Fig. 1). IL-8 also induced the mobilization of a molecule to the PMN surface, which was recognized by an antibody raised against clostridial neuraminidase but not by preimmune serum (Fig. 3). Functional changes in both PMN and peripheral blood mononuclear cells have been demonstrated with up to 30% desialylation (9, 38, 39). On the basis of the present studies, we cannot determine whether inhibition of endogenous sialidase activity altered PMN trafficking through modulation of global desialylation with changes in net surface charge, and/or by interfering with the removal of sialyl residues from specific glycoconjugates essential to PMN adhesion and migration, such as a β_2-integrin or the IL-8 receptor. We are currently examining the sialyl content of specific cell adhesion molecules following IL-8 treatment.

Since the removal of cell-associated sialic acid makes PMNs more adherent, deformable, and motile (11), a hypothetical schema may be proposed by which sialidase might regulate multiple steps in diapedesis. This enzyme shares the same intracellular compartment as cellular stores of CD11b/CD18. Sialidase may cleave the selectin (CD62L)-mediated low affinity adhesion to sialylated counterstructures that precedes the integrin-mediated tight adherence between PMNs and endothelium. Sialidase may also play a direct or indirect role in the functional activation of the integrins and/or the immunoglobulin-like adhesion molecules on endothelial cells (*e.g.* intercellular adhesion molecule 1) necessary for firm adhesion to occur. The mobilization of sialidase may facilitate the spreading of PMNs onto the adjacent endothelium, an essential step that precedes transendothelial migration. This may occur by removal of sialyl residues from specific, heavily sialylated molecules (*e.g.* leukosialin, also known as CD43) known to play a role in PMN adhesion to surfaces (40, 41). Alternatively, the desialylation of multiple glycoconjugate species on both the PMN and/or adjacent endothelial cell surface may sufficiently decrease the net negative surface charge of either or both cell types with a reduction of repulsive forces.

PMN movement to and its migration through the interendothelial cell junction requires reversible and dynamic changes in adhesion. This could occur through the alternating activities of sialidase and sialyltransferase enzymes. The translocated sialidase could desialylate cells adjacent to the activated PMN, perhaps along its leading edge, promoting PMN contact with the endothelial surface. Conversely, restoration of the preactivated state and release from the endothelium could rapidly occur if sialyltransferases (such as those previously described in human PMNs (42)) added sialyl residues back to glycoconjugates on the cell surface, perhaps at polarized sites on the PMN surface. Since sialidase is not released from the plasma membrane into the environment, this mechanism may localize the inflammatory response to the site of the responding cell. Thus, removal and restoration of sialyl residues from and to glycoconjugates on the surface may be a rapidly adaptable, highly orchestrated, yet spatially restricted process through which PMNs respond to chemotactic gradients across the endothelium without disrupting endothelial barrier integrity.

Therapeutic interventions targeting this enzyme may provide a novel strategy for limiting the

inflammatory response. There is an induction of sialidase and concomitant loss of sialyl residues from the PMN surface following stimulation with a wide range of agonists, including the chemokine, IL-8. Modulation of the activity of an endogenous mobilized sialidase by either antibody or a competitive inhibitor alters the trafficking of PMNs to the lungs in response to diverse stimuli. This strategy may have several advantages: by targeting multiple stages of diapedesis, inhibition of sialidase may provide a greater effect than may occur by inhibiting one step (*e.g.* the anti-selectin strategy (43)). Further, by inhibiting the effects of multiple, diverse chemotactic stimuli that are known to be generated in the lung, this approach may be preferable to targeting only one of these mediators (*e.g.* IL-8, leukotriene B4, or C5a). Since sialidase is not expressed on resting cells (Fig. 3) and since PMN sialidase remains bound to the activated cell, the anti-inflammatory effect of sialidase inhibitors should be restricted to activated PMNs and localized to sites of inflammation.

The presence of neuraminidase in diverse bacterial, viral, and parasitic pathogens may represent a form of molecular mimicry whereby these microbes take advantage of the pivotal role of surface sialic acid modulation by endogenous sialidase in immune responses and in cellular interactions described in this report. A similar dynamic modulation of surface sialic acid also may be critical to other processes requiring cell-to-cell contact, such as cell differentiation, antigen presentation, embryogenesis, and tissue remodeling as well as the metastatic potential of malignant cells. The potential therapeutic application of modulation of sialic acid content in these conditions merits further investigation.

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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: NANase, neuraminidase; PMN, polymorphonuclear leukocytes; ARDS, acute respiratory distress syndrome; fMLP, formyl-methionyl-leucyl-phenylalanine; PNP-NANA, 2-*O*-(*p*-nitrophenyl)α-D-*N*-acetylneuramic acid; 2-deoxy-NANA, 2,3-deoxy-*N*-acetyl-neuraminic acid; CVF, cobra venom factor; IL, interleukin; PIPES, 1,4-piperazinediethanesulfonic acid; FITC, fluorescein isothiocyanate; PNA, peanut agglutinin lectin; MPO, myeloperoxidase; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.

REFERENCES

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1. Cross, A. S., Asher, L., Seguin, M., Yuan, L., Kelly, N., Hammack, C., and Sadoff, J. (1995) *J. Clin. Invest.* **96**, 676-686[Medline] [Order article via Infotrieve]
2. Liu, D. Y., Petscheck, K. D., Remold, H. G., and David, J. R. (1982) *J. Biol. Chem.* **257**, 159-162 [Abstract/Free Full Text]
3. Lloyd, C. W. (1975) *Biol. Rev.* **50**, 325-350[Medline] [Order article via Infotrieve]
4. Wang, H., Klamo, E., and Kuhmann, S. (1997) *J. Virol.* **70**, 6884-6891[Abstract]
5. Witvliet, M. H., Burns, D. L., and Brennan, M. J. (1989) *Infect. Immun.* **57**, 3324-3330[Medline] [Order article via Infotrieve]
6. Ashwell, G., and Morrell, A. G. (1974) *Adv. Enzymol.* **41**, 99-128[Medline] [Order article via Infotrieve]
7. Gallin, J. I. (1980) *J. Clin. Invest.* **65**, 298-306[Medline] [Order article via Infotrieve]
8. Schauer, R. (1985) *Biochem. Sci.* **10**, 357-360
9. Cross, A. S., and Wright, D. G. (1991) *J. Clin. Invest.* **88**, 2067-2076[Medline] [Order article via Infotrieve]
10. Lichtman, M. A., and Weed, R. I. (1970) *Blood* **35**, 12-22[Medline] [Order article via Infotrieve]
11. Lichtman, M. A., and Weed, R. I. (1972) *Blood* **39**, 301-316[Medline] [Order article via Infotrieve]
12. Nydegger, U. E., Fearon, D. T., and Austen, K. F. (1978) *Proc. Natl. Acad. Sci.* **75**, 6078-6082 [Medline] [Order article via Infotrieve]
13. Roggentin, P., Schauer, R., Hoyer, L. L., and Vimr, E. R. (1993) *Mol. Microbiol.* **9**, 915-921 [Medline] [Order article via Infotrieve]
14. DePierre, J. W., Lazdins, J., and Karnovsky, L. (1980) *Biochem. J.* **192**, 543-550[Medline] [Order article via Infotrieve]
15. Kollef, M. H., and Schuster, D. P. (1995) *N. Engl. J. Med.* **332**, 27-37[Free Full Text]
16. Strieter, R. M., and Kunkel, S. L. (1994) *J. Invest. Med.* **42**, 640-651[Medline] [Order article via Infotrieve]
17. Baggiolino, M., Walz, A., and Kunkel, S. L. (1989) *J. Clin. Invest.* **84**, 1045-1049[Medline] [Order article via Infotrieve]
18. Torre, D., Zeroli, C., Giola, M., Fiori, P. G., Minoja, G., Marragia, D., and Chiaranda, M. (1993) *J.*

Infect. Dis. **167**, 505-506[Medline] [Order article via Infotrieve]

19. van Deventer, S. J. H., Hart, M., van der Poll, T., Hack, C. E., and Aarden, L. A. (1993) *J. Infect. Dis.* **167**, 461-464[Medline] [Order article via Infotrieve]
20. Carlos, T. M., and Harlan, J. M. (1994) *Blood* **84**, 2068-2101[Abstract/Free Full Text]
21. Huang, J., Hitt, N. D., and Kleinberg, M. E. (1995) *Biochemistry.* **34**, 16753-16757[Medline] [Order article via Infotrieve]
22. Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95-119[Medline] [Order article via Infotrieve]
23. Towbin, H., Stachelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350-4354 [Medline] [Order article via Infotrieve]
24. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual* , pp. 276-296, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Warren, L. (1959) *J. Biol. Chem.* **234**, 1072-1075
26. Milner, C. M., Smith, S. V., Carillo, M. B., Taylor, G. L., Hollinshead, M., and Campbell, R. D. (1997) *J. Biol. Chem.* **272**, 4549-4558[Abstract/Free Full Text]
27. Mulligan, M. S., Polley, M. J., Bayer, R. J., Nunn, M. F., Paulson, J. C., and Ward, P. A. (1992) *J. Clin. Invest.* **90**, 1600-1607[Medline] [Order article via Infotrieve]
28. Suzuki, K., Ota, H., Sasagawa, S., Sakatoni, T., and Fujikura, T. (1983) *Anal. Biochem.* **132**, 345-352[Medline] [Order article via Infotrieve]
29. Held, T. K., Mielke, M. E. A., Chedid, M., Unger, M., Trautmann, M., Huhn, D., and Cross, A. S. (1998) *Blood* **91**, 2525-2535[Abstract/Free Full Text]
30. DiMango, E., Zar, H. J., Bryan, R., and Prince, A. (1995) *J. Clin. Invest.* **96**, 2204-2210[Medline] [Order article via Infotrieve]
31. Mendla, K., and Cantz, M. (1984) *Biochem. J.* **218**, 625-628[Medline] [Order article via Infotrieve]
32. Craddock, P. R., Fehr, J., Brigham, K. L., Kronenberg, R. S., and Jacob, H. (1977) *N. Engl. J. Med.* **296**, 769-774[Abstract]
33. Ferrari, J., Harris, R., and Warner, T. G. (1994) *Glycobiology* **4**, 367-373[Abstract]
34. Miyagi, T., Hata, K., and Konno, K. (1992) *Tohoku J. Exp. Med.* **168**, 223-229[Medline] [Order article via Infotrieve]
35. Miyagi, T., Konno, K., Emori, Y., Kawasaki, H., Suzuki, K., Yasui, A., and Tsuki, T. (1993) *J. Biol. Chem.* **268**, 26435-26440[Abstract/Free Full Text]
36. Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., Morales, C. R., and Pshezhetsky, A. V. (2001) *J. Biol. Chem.* **276**, 46172-46181 [Abstract/Free Full Text]
37. Roggentin, P., Kleinedam, R. G., and Schauer, R. (1995) *Biol. Chem. Hoppe-Seyler* **376**, 569-575 [Medline] [Order article via Infotrieve]
38. Kelm, S., Shukla, A. K., Paulson, J. C., and Schauer, R. (1986) *Carbohyd. Res.* **149**, 59-64 [CrossRef][Medline] [Order article via Infotrieve]
39. Stamatos, N. M., Gomatos, P. G., Cox, J., Fowler, A., Dow, N., Wohlieter, J. A., and Cross, A. S. (1997) *Virology* **228**, 123-131[CrossRef][Medline] [Order article via Infotrieve]
40. Nathan, C., Xie, Q. W., Halbwachs-Mecarelli, L., and Jin, W. W. (1993) *J. Cell Biol.* **122**, 243-256 [Abstract]
41. Nong, Y. H., Remold-O'Donnell, E., LeBien, T. W., and Remold, H. G. (1989) *J. Exp. Med.* **170**, 259-267[Abstract]
42. Taub, R. N., Hindenburg, A. A., and Baker, M. A. (1985) *Leuk. Res.* **9**, 507-510[Medline] [Order article via Infotrieve]

43. Mulligan, M. S., Paulson, J. C., DeFrees, S., Zheng, Z. L., Lowe, J. B., and Ward, P. A. (1993) *Nature* **364**, 149-151[Medline] [Order article via Infotrieve]

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